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THE ACTIVITIES OF VARIOUS SUBSTITUTED PHENOLS IN STIMULATING THE RESPIRATION OF SEA URCHIN EGGS[1]

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Introduction

Whenever a series of related compounds is found to be capable of producing a given physiological effect, there always appears the possibility of relating the activities of the various substances to their molecular structure. In examining such possible relations it is important to distinguish between two different measures of the activity of a substance. Where the effect increases continuously with increasing concentration of the substance, the activities may be represented by the concentration required to produce an effect of a definite magnitude. But if the magnitude of the effect passes through a maximum with increasing concentration of the agent, the activity may be defined by the concentration required to produce the optimum (or maximum) effect or it may be defined by the magnitude of the optimum effect. It is also essential, when more than one molecular species of a substance exists, to determine which is the active form. Without such information no real comparison of activities can be made, since with different substances the concentration of the active form will depend upon the equilibrium conditions existing between the various molecular species.

Due principally to the work of Clowes and Krahl (1936) a large number of substituted nitro- and halophenols are now known that are capable of increasing the respiratory rate of marine eggs. They also exhibit the highly interesting reversible block to cleavage first demonstrated by these investigators (1934) with 4,6 dinitro-o-cresol. The effect of these agents is of further interest in that the increased respiratory rate is not accompanied by abnormal development, as is the case with the various oxidation-reduction dyes that had been previously (Barron, 1929; Runnström, 1930) shown to stimulate the

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respiration of marine eggs. These substances are then of interest to the embryologist since there exists the possibility of harnessing the extra energy that is liberated, and the ability to stop cleavage in a reversible manner should help us discover the factors that cause it to go.

The substituted phenols are all weak monobasic acids and the question of the active molecular species relates to whether it is the anion or the undissociated molecule that is effective. In a previous publication (1937) we have presented evidence for the view that it is the anion that is the active form inside the cell, but that penetration must be effected as the undissociated molecule. The evidence is based in part on the existence of a pH effect. At lower pH's it requires a lower concentration of a given substituted phenol to produce the maximum respiratory stimulation or reversible block to cleavage than it does at higher pH values of the solution. But the calculated concentration of undissociated molecules is very closely the same at the different pH's. Field et al. (1934) first demonstrated this pH effect of 2,4 dinitrophenol on yeast. He interpreted the result to mean that the substance is active as the undissociated molecule. However, when we compared a number of different nitro- and halophenols on the basis of the concentration of undissociated molecules present at maximum effective total concentration, enormous differences were found. It appeared for the extremes that a million molecules of one substance would be required to produce the same effect as one molecule of another if the undissociated molecule were the active form. On the other hand, when the concentration of the anion inside the cell was estimated, assuming that the substances penetrate freely in the undissociated form, and using the value for the internal pH determined by Chambers and Pollack (1927) and the Needhams (1926) a more reasonable result was obtained. The different compounds then gave values of the same order of magnitude.

The calculations of the concentrations of the undissociated molecules and of ions in the solution and inside the cell are made from the usual mass action equations. The concentration of undissociated molecules, $[HP]$, present in the solution may be expressed as a function of the hydrogen ion concentration, $[H^+]$, and the total concentration, $[\Sigma P]$, by the equation:

$$[HP] = \frac{[H^+][\Sigma P]}{[H^+] + K}, \tag{1}$$

where K is the constant for the acid dissociation of the particular substituted phenol and $[\Sigma P] = [HP] + [P^-]$. The concentration of anions, $[P^-_i]$, inside the cell is given simply by the equation:

$$[P^-_i] = \frac{[HP_i]\,K}{[H^+_i]} \tag{2}$$

in which the subscript, i, refers to values for the inside of the cell. There are several assumptions involved here. Firstly, the value of $[HP_i]$ is taken to be the same as $[HP]$ in the solution. This follows from the evidence that the substituted phenols penetrate freely in the undissociated form. Secondly, it is assumed that activity coefficients may be neglected. The error involved in this assumption is certainly very small in comparison with other sources of error in the data. It is assumed, thirdly, that $[H^+_i]$ is not changed by the addition of these substituted phenols. We shall discuss this point below.

Combining equations (1) and (2) we have:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]\,K}{[H^+_i]([H^+_o] + K)}, \tag{3}$$

where the subscript, o, refers to the solution and the subscript, i, to the inside of the cell. In the cases where K is large with respect to $[H^+_o]$ then $[P^-_i]$ will vary directly with the hydrogen ion concentration of the solution and with total concentration of substituted phenol, namely:

$$[P^-_i] = \frac{[H^+_o][\Sigma P]}{[H^+_i]}. \tag{4}$$

Where K is small with respect to $[H^+_o]$ we have

$$[P^-_i] = \frac{[\Sigma P]\,K}{[H^+_i]}. \tag{5}$$

In the situation represented by equation (4), $[P^-_i]$ is independent of K. Therefore those compounds having relatively large values of K will give the same value of $[P^-_i]$ when $[\Sigma P]$ and $[H^+_o]$ are the same. $[HP]$ at the same time will vary inversely with K as may be seen from equation (1). In the situation represented by equation (5), $[P^-_i]$ is independent of $[H^+_o]$. Therefore those compounds with small values of K will show a direct proportionality between $[P^-_i]$ and K for the same $[\Sigma P]$. $[HP]$ at the same time will be practically equal to $[\Sigma P]$. In either situation, then, $[P^-]$ and $[HP]$ will vary in a different manner, so it will not matter whether we investigate substances with large or with small dissociation constants, provided that the different compounds have sufficiently different dissociation constants. In other words, we could not hope to determine the active form if all the members of the series had very closely the same dissociation constant.

If the dissociation constants for the different substituted phenols were all small or all large we would want to know their values very exactly. But since in our series there are compounds with large values as well as small values available, this is not so essential.

The experiments reported here are an extension of previous work (1937) and involve a comparison of the activities of various substituted nitro- and halophenols. We employ here the first criterion of activity referred to above; namely, the concentration required to produce a definite effect. Data on the magnitude of the maximum stimulation are also presented. In addition a new type of experiment is reported in which the internal pH is altered, and the effect on the optimum concentration determined. Also the buffer capacity of eggs is estimated.

Material and Methods

The eggs of the sea urchins, *Strongylocentrotus purpuratus* (at Corona del Mar, California) and *Arbacia punctulata* (at Woods Hole, Massachusetts), were used in these experiments. These two forms behaved very much alike with respect to the concentrations of the substituted phenols required for maximum respiratory stimulation and reversible cleavage block as well as the magnitude of stimulation.

The oxygen consumption measurements were made by means of the Barcroft-Warburg manometers using the conical type of vessel with side-arm (Anhang). The substituted phenols are added from the side-arm after a preliminary series of readings (lasting usually one hour) on the untreated eggs. The solutions were buffered by means of glycylglycine, which we have recently (1937) shown to be highly suitable for buffering in the neighborhood of pH 8.0. The concentration of glycylglycine was generally 0.015 molar in carbonate-free sea water, prepared as previously described. The pH's were determined by means of the glass electrode (Beckman pH meter), the values for the egg suspensions in the vessels at the end of a run being taken as well as that of the initial solutions.

Results

The nitro- and halophenols [2] listed in Table I were investigated. In all cases a series of at least six different concentrations were employed in order to determine the one giving maximum stimulation. From the theoretical considerations based on the assumption that the

[2] The dichlorophenols were generously supplied by Dr. John Ruhoff of the Mallinckrodt Chemical Works, and were subsequently purified and their melting points checked. The other compounds were obtained from the Eastman Kodak Company.

substances are active as the anion, $[P^{-}_{i}]$ inside the cell, it was possible to determine what total concentration to employ at a given pH of solution. Thus a fairly closely graded series of concentrations could be used, which, if the assumptions were correct, should include that at which maximum respiratory stimulation is obtained.

The criterion for maximum respiratory stimulation involves a point which has not been emphasized in previous work with substituted phenols. With increasing concentration the respiratory rate

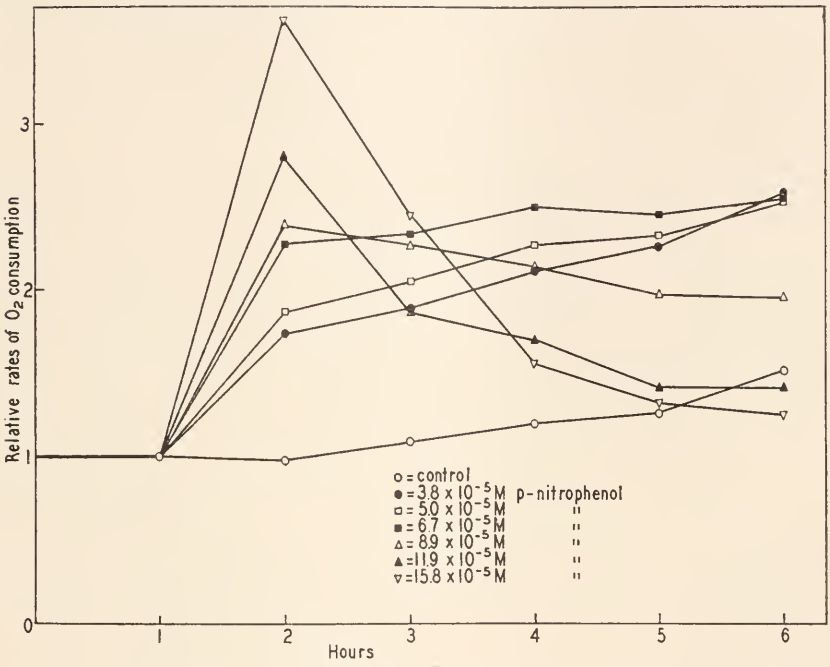

FIG. 1. Rates of oxygen consumption of eggs of *Strongylocentrotus purpuratus* in various concentrations of p-nitrophenol in glycylglycine-buffered sea water at pH 7.90. The reagent is tipped into the egg suspension at the end of the first hour. The relative rates of oxygen consumption are given on the basis of the oxygen consumption during the first hour as unity. Measurements begun at 43 minutes after fertilization. Temperature = 20° C.

increases, but beyond a certain concentration the increase does not remain constant with time. There is, at these higher concentrations, an initial rise in respiratory rate lasting for a short period of time followed by a steady drop in rate, which may finally reach values below that of the controls. This can be seen in Fig. 1, in which the rates of oxygen consumption in different concentrations of p-nitrophenol are plotted against time. With concentrations up to 6.7×10^{-5} molar the rate rises with time as does the control (untreated eggs) rate during

this interval. But above this concentration there is an initial increase in rate followed by a steady drop, which in the case of the two highest concentrations, falls at the end of 6 hours to a value below that of the controls. This kind of variation of the oxygen consumption rates with time is found with the higher concentrations of all the substituted phenols that we have investigated. It is evident, with concentrations giving this initial rise followed by a decrease, that the particular value taken to represent the rate will depend upon the length of time after addition of the reagent during which the respiration is followed. One cannot, then, very well specify the amount of stimulation at these higher concentrations. We shall therefore take as the optimum concentration that concentration giving the greatest increase in rate of respiration without a subsequent decrease.

At the optimum concentration defined in this way we find that cleavage proceeds normally. The reversible cleavage block appears at concentrations just above this optimum; i.e., at concentrations at which there is an initial stimulation followed by a decrease. Thus in the experiment illustrated in Fig. 1, cleavage was blocked in the three highest concentrations of p-nitrophenol. Examination of the eggs from the vessels one hour after the end of the run gave the following percentages in the various stages:

	Number of Cells					Early blastulae
	1	2	4	8	16	
Control	1	1	0.5	0.5	0.5	96.5
3.8×10^{-5} M	1	1	0.5	0.5	0.5	96.5
5.0×10^{-5} M	1	1	0.5	0.5	0.5	96.5
6.7×10^{-5} M	1	1	0.5	0.5	0.5	96.5
8.9×10^{-5} M	1	1	0.5	30	64.5	3
11.9×10^{-5} M	1	39	60	0	0	0
15.8×10^{-5} M	1	89	10	0	0	0

The p-nitrophenol was added 15 minutes before the eggs were due to go into 4 cells. There is, as the data show, a time lag before cleavage is blocked which is particularly evident with the weakest blocking concentration. With higher concentrations the block is more immediate. At the same time the initial rise in respiratory rate is of much shorter duration in such solutions.

Using as the optimum concentration that which gives the maximum stimulation without a subsequent decrease with time, we may compare the various substituted phenols. In Table I these optimum concentrations, $[\Sigma P]$, at pH 8.0, are given for 15 substituted phenols. The

amount of respiratory stimulation at the optimum is also listed. We shall not at this time attempt to compare the various compounds on the basis of magnitude of respiratory stimulation. The comparison on the basis of optimum concentrations must, as was previously pointed out, take into account the pH effect which showed that for

TABLE I

Concentrations required for optimum stimulation of rate of oxygen consumption in fertilized sea urchin eggs. Temperature = 20.0° C.
$[\Sigma P]$ = total concentration in solution at pH 8.0.
$[HP]$ = calculated concentration of undissociated molecules.
$[P^-_i]$ = calculated concentration of the anion inside the cell.

	$[\Sigma P]$	Max. resp. stimulation	pK	$[HP]$	$[P^-_i]$
	$M \times 10^5$	*per cent*		$M \times 10^{10}$	$M \times 10^6$
o-nitrophenol	373	37(?)	7.25†	2,780,000	62.2
m-nitrophenol	27.3	93	8.34†	1,870,000	3.4
p-nitrophenol	10.3	128	7.22†	146,000	3.5
2, 4-dinitrophenol	6.78	172	4.00†‡	67.8	2.7
2, 6-dinitrophenol	11.6*		3.59†	45.3	4.6
2, 4, 6-trinitrophenol	1090*	0	0.80‡	6.9	440
o-chlorophenol	47.2*		8.50§	3,590,000	4.5
m-chlorophenol	69.9	71	8.85§	6,130,000	3.5
p-chlorophenol	181	30	9.18§	17,000,000	4.5
2, 4-dichlorophenol	23.5	131	7.75§	876,000	6.2
2, 5-dichlorophenol	12.3	73	7.35§	224,000	3.6
2, 6-dichlorophenol	991*	0	6.80§	5,880,000	371
3, 5-dichlorophenol	20.8	39	7.92§	943,000	4.5
2, 4, 6-trichlorophenol	6.9*		6.00†	6,870	2.7
2, 6-dichloro-4-nitrophenol	4.87	140	3.70‖	24.4	2.0

* Value determined from cleavage block data, i.e., concentration just below blocking.
† From the International Critical Tables.
‡ From Scudder (see bibliography).
§ From Murray and Gordon (see bibliography).
‖ From Bader (see bibliography).

a given substituted phenol, the same effect (e.g., optimum respiratory stimulation) was obtained at different pH's when there was the same calculated concentration of undissociated molecules present. The comparison of the different compounds is therefore first made on the basis of the concentration of undissociated molecules, $[HP]$, present in the solution at total concentrations giving optimum respiratory stimulation. These values are listed in the fourth column of the table, and it is evident that there are enormous differences between the different compounds. If the undissociated form were considered to be active (as the pH effect might indicate) then it would mean, for ex-

ample, that 700,000 molecules of p-chlorophenol are required to do the same job as one molecule of 2,6-dichloro-4-nitrophenol. Since this appeared extremely unlikely, a comparison of the various compounds was made on the basis of the calculated concentrations of anions, $[P^-_i]$, inside the cell at total concentrations giving optimum respiratory stimulation. The assumptions involved in this calculation are given in the introduction. These values are listed in the last column of Table I and it may be seen that with the exception of three compounds the values are all of the same order of magnitude. Two of the exceptions; namely, 2,4,6-trinitrophenol and 2,6-dichlorophenol give no respiratory stimulation, the $[P^-_i]$ values being obtained from cleavage block data. The other exception, o-nitrophenol, gave a small stimulation of respiratory rate. But our sample of this compound was probably impure as judged by its melting point. Dr. Clowes has suggested that the impurity may well be p-nitrophenol, which would account for the slight respiratory stimulation.

The three exceptions may therefore be omitted since they are not respiratory stimulants. The same may possibly hold for o-chlorophenol which was not sufficiently investigated to determine whether or not it will stimulate the respiratory rate. The narrow range in which the values of $[P^-_i]$ lie contrasts strikingly with the great divergence in $[HP]$ values of the various substituted phenols. Of the two alternatives, the anions $]P^-_i]$ or the undissociated molecules $[HP]$, the evidence shows that it is the former that must be considered the active form inside the cell.

The calculated $[P^-_i]$ values for different substituted phenols vary, the extremes showing a threefold variation. The data, however, are not sufficiently accurate for the differences to be considered significant. There are various sources of errors in the data. The errors in the manometric method are probably negligible. Those involved in the pH determinations and in slight variations in the course of a run are negligible for compounds with high pK values but not for compounds with low pK values. The pK values which are taken from the literature (see superscripts in Table I) are themselves subject to some error. This, as was shown in the introduction, affects principally the $[P^-_i]$ values for those compounds with high pK. In addition, certain of these compounds, particularly the mono- and dichlorophenols, are rather volatile, so that during the course of a run a certain amount of distillation into the alkali well of the vessel occurs. The magnitude of this factor is difficult to assess. It would, of course, tend to make the calculated $[P^-_i]$ values too high.

Effect of Changing the Internal pH

If the anion is the active form within the cell, then by increasing the internal pH (keeping the external pH constant) a lower total concentration of the substituted phenol should be required to give the optimum respiratory stimulation or the reversible block to cleavage. It is possible to increase the internal pH by the addition of a weak penetrating base such as ammonia. The pH change is readily demonstrated by first staining the eggs with neutral red, and, after washing, immersing them in the ammonia solution. The extent to which the red color fades (neutral red changes from red to yellow between pH 6.8 and pH 8.0) gives the increase in internal pH that has occurred.

The effect of increasing the internal pH on the concentration of dinitrophenol required for reversible cleavage block was investigated with eggs of *Urechis*. The solutions were prepared from carbonate-

Table II

Cleavage inhibition of Urechis eggs with 2, 4-dinitrophenol in ammoniacal sea water. Experimental and control solutions both prepared from carbonate-free sea water and buffered at pH 8.43 with glycylglycine.

Concentration of 2, 4-DNP	Cleavage in ammonia solution	Cleavage in control solution
(Molar)	(*per cent*)	(*per cent*)
0	99	99
2.6×10^{-4}	95	95
3.9×10^{-4}	0	95
6.0×10^{-4}	0	90

free sea water using the glycylglycine buffer and adjusting the pH with ammonia. In the control solutions NaOH was used instead of ammonia. It was found in the first place, that when sufficient ammonia was present to effect an increase in internal pH, cleavage was interfered with or proceeded abnormally. These same solutions when used on unfertilized eggs gave artificial parthenogenesis. With solutions weak enough in ammonia to have no effect on cleavage and no activating effect on unfertilized eggs there was no observable effect on the internal pH. We were unable to prepare an ammonia solution which would give an observable increase in internal pH without at the same time affecting cleavage or activating unfertilized eggs.

It was possible, however, to demonstrate, with the weakest ammonia solutions that give an observable increase in internal pH, an effect on the concentration of dinitrophenol required for cleavage block. The results of such an experiment are given in Table II. The

percentage of eggs that divided in the ammonia solution is seen to be as high as in the control. The cleavage here was rather abnormal (in regard to size of blastomeres) and only abnormal bottom-swimming embryos were obtained as compared with 90 per cent top swimmers in the controls. It may be seen from the table that the concentration of dinitrophenol required to block cleavage is considerably less in the ammonia solution than in the control. This result conforms then to the expectation, on the basis of the anion being the active agent inside the cell. The fact that the ammonia solution employed did not give normal later development does not seriously interfere with the interpretation, since we would not expect a summation effect with dinitrophenol.

Buffer Capacity of Eggs

One of the assumptions on which the calculation of the concentration of anions inside the cell is based is that the internal pH of the cell does not change in the solutions used. Since the substituted phenols are weak acids and penetrate only in the undissociated form,[3] an acidification of the interior might be expected to occur. This would be independent of the pH of the external solution and the extent of acidification should depend upon the total concentration of the substance. The amount of decrease in internal pH that would be expected would also depend upon the buffer capacity of the egg and the dissociation constant of the particular substituted phenol. We have attempted to settle this point by staining eggs with indicators that have their color change in the region of pH 4 to 6, but have been unable to find any that penetrate readily.

In place of this, a determination of the buffer capacity of egg material was made. For this determination eggs of *Strongylocentrotus* were first frozen at − 72° C. for several hours and rapidly thawed to room temperature. This completely breaks up the eggs, resulting in a fine granular suspension which we shall refer to as egg-brei. An egg brei containing originally 2 parts of eggs to one part of sea water by volume showed a pH of 6.35. In order to allow the suspension to be more easily stirred during titration, it was diluted with an equal volume of distilled water, which resulted in a slight rise in pH to 6.41. The titration curve of this diluted egg brei is shown in Fig. 2 (curve *A*), and a titration curve of ordinary sea water is given for comparison (curve *B*). In the 10 cc. of egg brei that was titrated there was $1\frac{2}{3}$ cc. of sea water along with the $3\frac{1}{3}$ cc. of egg material. Comparison

[3] Beck and Chambers (1937) have recently presented evidence to show that salts of weak fatty acids can under certain conditions penetrate marine eggs.

of curves *A* and *B* (the latter is for 5.0 cc. of sea water) shows that the correction for the sea water present is negligible. The buffer capacity of the egg brei is evidently quite high as the figure shows. To lower the pH from 6.4 to 5.4, 1.65 cc. of 0.1 normal acid are required. For the same buffering action a univalent acid with a pK of 6.4 would have to be present in an initial concentration of 0.04 molar, or considering the eggs alone, 0.12 molar.

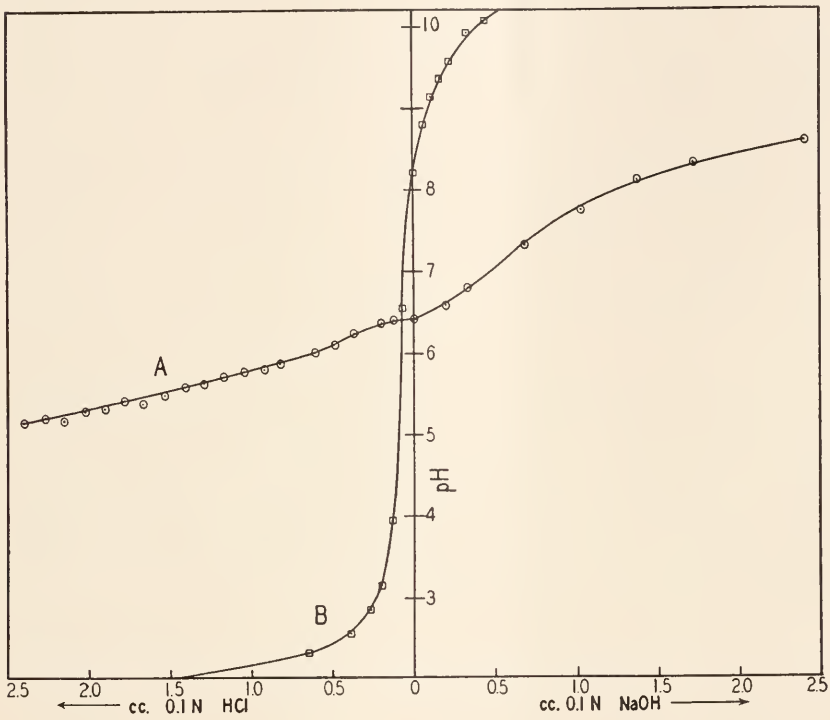

FIG. 2. Curve *A;* titration of 10 cc. of a suspension of sea-urchin egg brei (prepared by freezing at −72° C.) containing 3⅓ cc. of egg material, 1⅔ cc. of sea water, and 5 cc. of distilled water. Curve *B*; titration of 5 cc. of sea water.

We cannot, of course, assume that the buffer capacity determined in this manner gives a true picture of the situation in the living egg. Nevertheless, it would not be expected to be radically different. It is interesting to note that the egg brei shows a pH of 6.4 which is not very different from the value of 6.6 obtained by micro-injection of indicators (Needham and Needham, 1926).

For raising the internal pH with ammonia, at an external pH of 8, the total concentration of ammonia must be greater than 0.01 molar.

The optimum *total* concentrations of the different substituted phenols are all much lower than this.

It may be concluded, then, that the egg is sufficiently buffered to maintain a fairly constant pH in the presence of the concentrations of the substituted phenols employed in these experiments.

Discussion

In two recent articles, Krahl and Clowes (1938) present some tentative conclusions of their investigations on the action of substituted phenols. They are in agreement with our view (T. and H., 1937*b*) that penetration is effected in the undissociated form. Also, respiratory stimulation below the optimum is stated to depend upon the anion concentration inside the cell. But cleavage block and initial respiratory decrease (beyond the optimum), is considered to depend upon the concentration of undissociated molecules. The amount of stimulation at the optimum is then, according to these investigators, dependent upon the concentration of anions attained when the limiting concentration of molecules is reached, in the case of any given substituted phenol. The data obtained by Krahl and Clowes and by us agree, on the whole, very well. But in the experiments on the effect of changing the internal pH of the cell we differ. They find practically no effect on the total concentration required for cleavage block, whereas we find an effect in the direction expected according to our interpretation. Since the evidence from this source is partly responsible for the divergence in our respective views, a thorough investigation of the effect of changing the internal pH would be desirable. The difficulties involved in such experiments have been indicated above.

Field, Martin, and Field (1935, 1936) have compared the activity of several substituted phenols in stimulating yeast respiration. With those compounds for which dissociation constants were available, the calculated optimum concentrations of the undissociated form are given, since that is considered the active form by these authors. We have calculated from their data the concentration of anions that would be present inside the cell, using Gutstein's (1933) value of 6.2 for the pH of yeast. These values differ almost as much as do the figures for the optimum concentration of the undissociated form of the different substituted phenols that they list. It appears then that the situation in yeast is different from that in the marine eggs. It may be noted that the optimum concentrations of undissociated molecules obtained with yeast are much higher than with eggs, ranging from about 10 times as much in the case of the mono-nitrophenols to 1000 times as much with the dinitrophenols. It is not feasible at present to attempt

to resolve the differences between yeast and marine eggs. It is evident, however, that the wide differences in [*IIP*] obtained with the various substituted phenols in yeast do not support the view that the undissociated form is active.

Recently Bodine and Boell (1938) have reported experiments on grasshopper embryos in which no pH effect is manifest with suboptimal concentrations of dinitrocresol. Actually their curves show a slight increase in respiratory stimulation as the pH is lowered, but the effect is much too small to be interpreted simply on the basis of the variation in concentration of undissociated molecules. Possibly in the grasshopper egg the changes in external pH produce proportionate changes in internal pH. This would, in the case of substituted phenols with relatively large dissociation constants (see page 211) tend to result in a constant or only slightly varying $[P^-_i]$ and would, according to our view, explain the absence of a pH effect on the respiratory stimulation. The more marked effect which they obtained with a stronger than optimum concentration might be similarly explained, especially since the stimulation drops off quite rapidly with concentrations above the optimum.

The differences in $[P^-_i]$ shown in Table I do not seem to us to be significant, for the reasons stated above. It appears likely, then, that the different substituted phenols have the same activity, considered on the basis of the $[P^-_i]$ required to produce the same effect. This is consistent with the view previously expressed by Clowes and Krahl (1936) that these compounds owe their activity to the phenolic OH group. The magnitude of the respiratory stimulation would depend then on the effect on the phenolate ion of the type of substitution in the benzene ring.

Summary

1. Data on the concentrations required for optimum respiratory stimulation and reversible cleavage block in fertilized sea urchin eggs are presented for fifteen different nitro- and halophenols.

2. The experiments show that it is necessary to define the optimum as the highest respiratory increase that does not diminish with time.

3. Cleavage block occurs with concentrations just beyond the optimum defined in this manner.

4. Comparison of the calculated concentrations of undissociated molecules and of anions inside the cell for the different substituted phenols supports the previously expressed view that the anion is the active agent. Penetration is accomplished in the undissociated form, as previously shown.

5. The results of experiments in which the internal pH is raised also support this view.

6. Titration curves of egg brei indicate a high buffer capacity for the egg and support the assumption that the internal pH does not change in the presence of the substituted phenols.

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